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Quantifying Biomarkers of Liver Damage Using Shotgun Proteomics

Lee W. Ott
Frank Witzmann
Indiana University School of Medicine
Indianapolis IN 46151

Camilla A. Mauzy
Claude C. Grigsby
Deirdre A. Mahle
John J. Schlager

Biosciences and Protection Division
Applied Biotechnology Branch
Wright-Patterson AFB OH 45433-5707

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______//SIGNED//__________
Diane Todd, Work Unit Manager
Applied Biotechnology Branch

________/ /SIGNED// _______
Mark M. Hoffman, Deputy Chief
Biosciences and Protection Division
Human Effectiveness Directorate
Air Force Research Laboratory

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* Lee W. Ott, Frank Witzmann

** Camilla A. Mauzy, Claude C. Grigsby, Deirdre A. Mahle, John J. Schlager

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14. ABSTRACT
In a preliminary examination of labeless protein quantitation proteomics, we investigated the serum composition of rats treated with the hepatotoxin alpha-naphthylisothiocyanate (ANIT) to find biomarkers of exposure. ANIT was administered orally to the rats in doses of 1, 20, and 100 mg/kg. Collected serum was immunodepleted and analyzed using an LTQ mass spectrometer. MS/MS spectra were searched against the rat IPI protein database to identify peptides, and valid peptides were grouped according to protein and quantified using Bioworks v3.2. In examining the dataset, only the proteins with the highest abundance relative to the control population were considered. T-kininogen and other kininogen family proteins were found to increase on average of 3-5 fold in all three treatment groups compared with the control. Complement C9 protein averaged 3- to 6-fold higher versus control. Since ANIT is an accepted model of intrahepatic cholestasis, the data from this study may help indicate cholestasis status.

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PREFACE

Special acknowledgement goes to Nathan Pedrick in Frank Witzmann's laboratory for assistance in instrumentation. This research was supported in part by an appointment to the Research Participation Program at the Air Force Research Laboratory, Human Effectiveness Directorate, Bioscience and Protection Division, Wright-Patterson AFB OH, administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and AFRL/RHP.

INTRODUCTION

Alpha-Naphthylisothiocyanate (ANIT) is a widely used model hepatotoxin for intrahepatic cholestasis [1-3]. Although the mechanism of ANIT-induced hepatotoxicity is not completely understood, the current model is a reversible glutathione-ANIT conjugate is produced in hepatic parenchymal cells and selectively accumulated in bile where it dissociates to reduced glutathione and ANIT [4, 5]. The exposure of biliary epithelial cells to a cytotoxic level of ANIT results in necrotic death followed by hyperplastic growth and eventually the obstruction of bile flow. At some point, there is an increase in the permeability of the tight junctions between parenchymal cells and bile duct epithelial cells resulting in the exposure of parenchymal cells to a necrotic concentration of bile acid salts. Neutrophil infiltration and subsequent release of free radicals and serine proteases from the infiltrated neutrophil have been implicated in ANIT hepatotoxicity [6, 7]. Reactive oxygen species have also been implicated as a mechanism of ANIT-induced cholangiocyte apoptosis [8], and disruption of lipid homeostasis contributes to ANIT-induced cholestasis [9, 10].

This study aims to determine biomarkers of liver damage induced by ANIT. These biomarkers have the potential to detect the severity of cholestasis. Proteins increased in response to low levels of ANIT could be indicative of mild cholestasis; while the higher level biomarkers could represent more severe cholestasis.

MATERIALS AND METHODS

Male Fischer 344 rats were orally gavaged with 0, 1, 20, or 100 mg/kg of ANIT in corn oil. Sera were collected 96 h post-dose, and albumin, IgG and transferrin were depleted using an Agilent multiple affinity removal cartridge specific for mouse and rat. Samples were dried and resuspended with 8M Urea/100mM Ammonium Bicarbonate buffer. A Bradford protein assay was used to determine protein concentration. Proteins were reduced with dithiolthreitol (40-fold molar excess), alkylated with iodoacetimide (70-fold molar excess), and subjected to an overnight tryptic digest (1:70 trypsin:protein). 10cm of 5 micron particle size C-18 reverse phase resin (Michrom Biosciences, Inc.) was packed into 100 micron ID fused silica tubing using a pressurized bomb. The column was placed inline with the Paradigm HPLC System (Michrom Biosciences, Inc.). Peptides were loaded onto the C-18 resin using the autosampler and eluted over 95 minutes using a gradient of Acetonitrile (5% to 60% Acetonitrile in 0.025% Formic Acid) and sprayed directly into an LTQ mass spectrometer. Sequest was then used to search the MS/MS to find the best theoretical match for each experimental spectrum. Bioworks 3.2 filtered on the basis of XCorr values (+1 ions 2.56, +2 ions 3.22, +3 ions 3.45) and integrated the peak area of each peptide and grouped the peptides into proteins. Protein ratios were then determined using normalized peak areas (peak area/total peak area multiplied by 100). Each sample was analyzed three times by mass spectrometry to account for technical variance.

RESULTS

Protein levels decrease upon exposure to increasing levels of ANIT Table 1. All ratios shown as ANIT treated over pretreated and represent an average of three independent analyses. These proteins would be indicative of healthy individuals not exposed to the hepatotoxin.

Table1. Proteins that Decrease in Response to ANIT. The second column lists the IPI protein database accession number. The last three columns represent doses of ANIT.

Protein Name	IPI	1 mg/kg	20 mg/kg	100 mg/kg
Alpha-1-inhibitor 3 precursor	IPI00201262	0.946767	0.485953	0.013578
Alpha-2-globin chain	IPI00205036	0.456655	0.14163	0.083014
Alpha-2-HS-glycoprotein precursor	IPI00327469	1.565994	0.861849	0.169885
Anionic trypsin I precursor	IPI00212767	2.425354	0.124566	0.058506
Apolipoprotein A-I	IPI00563778	1.485341	0.540123	0.468427
Apolipoprotein A-I precursor	IPI00197703	1.485341	0.540123	0.468427
Apolipoprotein A-IV precursor	IPI00324272	0.538851	0.681997	0.181877
Contrapsin-like protease inhibitor 1 precursor	IPI00200593	1.453088	0.597673	0.305622
Contrapsin-like protease inhibitor 3 precursor	IPI00200591	1.308563	0.592263	0.209985
Fetub protein	IPI00212708	0.70911	0.921711	0.15584
Fetuin-B precursor	IPI00559588	0.70911	0.921711	0.152683
Hemoglobin alpha-1/2 subunit	IPI00287835	0.456655	0.14163	0.083014
Histidine-rich glycoprotein	IPI00191789	2.128469	1.059675	0.311549
Histidine-rich glycoprotein 1	IPI00201347	1.748193	1.063261	0.388458
Murinoglobulin 2	IPI00564327	1.122217	0.385506	0.049461
PREDICTED: hypothetical protein XP_579477	IPI00734558	0.972352	0.600282	0.00734
PREDICTED: similar to Murinoglobulin 1 homolog	IPI00368704	1.121146	0.455815	0.020759
Rat alpha(1)-inhibitor 3, variant I precursor	IPI00212666	0.977366	0.61013	0.007432
Serine protease inhibitor 2.1 (Fragment)	IPI00211074	1.011942	0.403521	0.095942
Vitamin D-binding protein precursor	IPI00194097	0.658084	0.299516	0.083001

Protein levels increased in exposure to increasing doses of ANIT Table 2. The protein ratios are expressed as ANIT treated over pretreated and represent an average of three independent analyses. Proteins in this table represent putative biomarkers to exposure of ANIT.

Table 2. Potential biomarkers of exposure to ANIT. The second column lists the IPI protein database accession number. The last three columns represent doses of ANIT.

Protein Name	IPI	1 mg/kg	20 mg/kg	100 mg/kg
Complement component 9	IPI00561894	3.83715	2.981348	6.184085
Fibrinogen, gamma polypeptide	IPI00555210	0.409077	3.320876	2.540892
Haptoglobin precursor	IPI00325610	0.007605	0.278047	0.342858
Inter-alpha-trypsin inhibitor heavy chain H3 precursor	IPI00326984	0.23479	0.537671	0.414187
Rat T-kininogen	IPI00187796	3.005327	4.389969	3.32364
Splice Isoform 1 of Fibrinogen alpha chain precursor	IPI00202651	0.695303	4.514717	2.074696
Splice Isoform Gamma-A of Fibrinogen gamma chain precursor	IPI00230944	0.337093	3.414798	2.458402
Splice Isoform of Fibronectin precursor	IPI00200757	0.010109	0.53244	0.497446
T-kininogen I precursor	IPI00327182	2.291727	4.389969	3.32364
T-kininogen II precursor	IPI00679245	2.284642	6.508513	11.90976

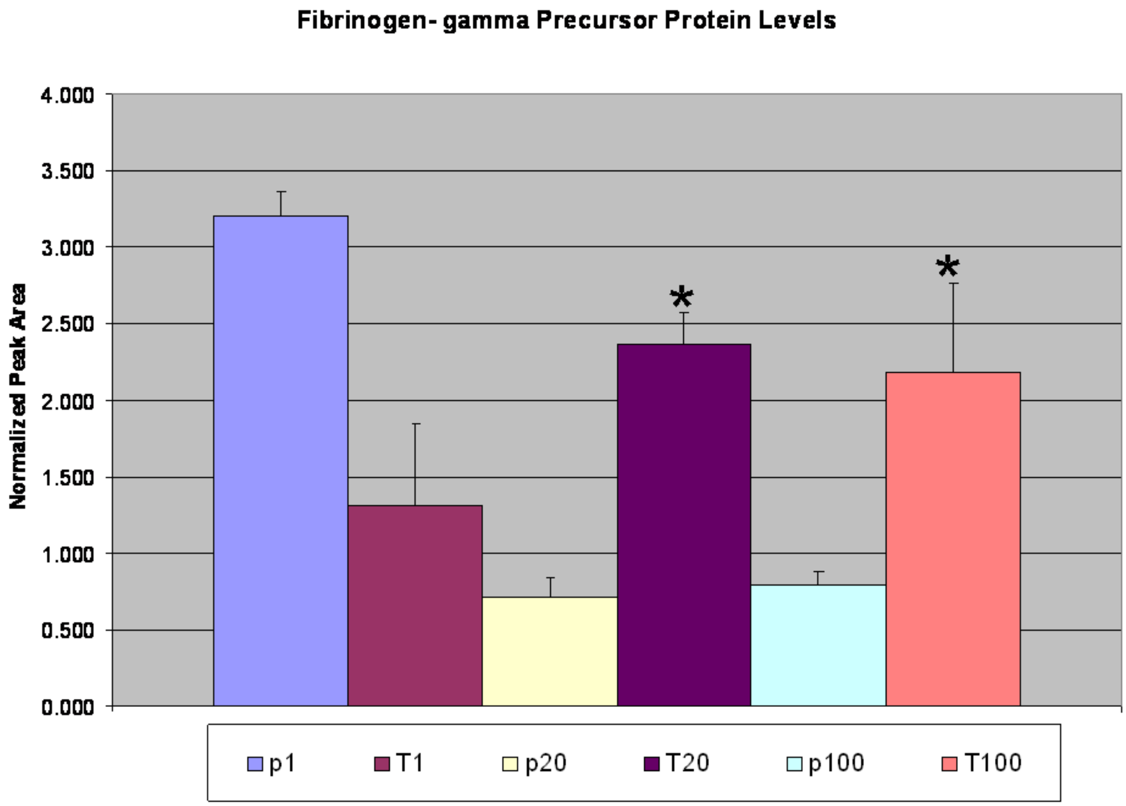

Figure1. **Fibrinogen Levels.** Fibrinogen represents a putative biomarker of higher doses of ANIT. *Only at the 20 mg/kg and the 100 mg/kg doses are the protein levels significantly higher ($p<0.05$). p = pretreated (control), T = treated; 1, 20, 100 = 1, 20, or 100 mg ANIT per kg body weight

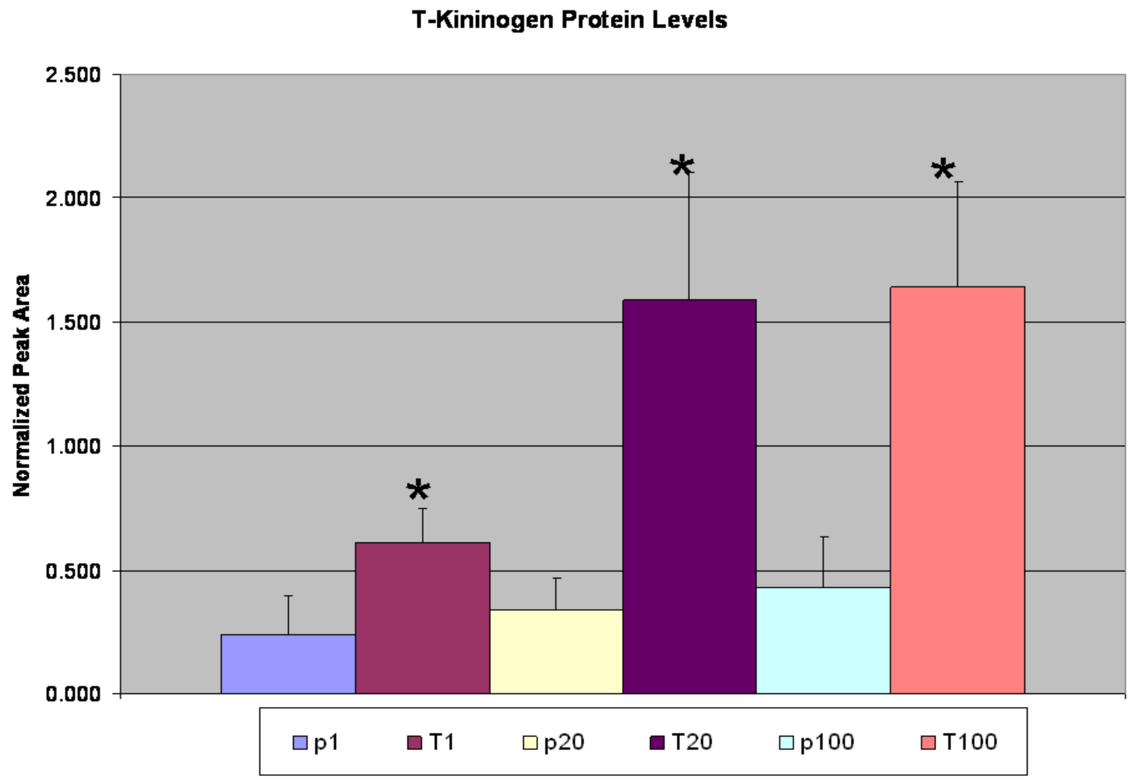

Figure 2. **Potential biomarker of ANIT.** Kininogen represents a putative biomarker of ANIT. *For all three doses of ANIT the protein levels of kininogen are significantly higher ($p<0.05$) than the protein levels in the pretreated rats. p = pretreated (control), T = treated; 1, 20, 100 = 1, 20, or 100 mg ANIT per kg body weight

DISCUSSION

The proteins induced by ANIT treatment play a role in regulating the blood clot formation. Kininogen has no enzymatic activity and plays a role in initiating the intrinsic coagulation pathway [11]. Negatively charged phospholipids have been shown to initiate the formation of blood clots [12, 13]. ANIT has been shown to increase the serum concentration of these phospholipids [10]. This event could mimic the natural release of phospholipids by blood platelets. Sera levels of serine proteases also increase in response to ANIT [6, 7, 14]. Serine proteases play an essential role in the conversion of fibrinogen precursors to active fibrin. Fibrin polymers solidify the clot. Although serine proteases were not detected in any of the treatment groups, serine protease inhibitors (murinoglobulin and serine protease inhibitor 2.1) and the alpha-1 inhibitor 3 precursor protein levels decrease in response to increasing levels of ANIT. Since serine protease activity is essential for proper clotting, low levels of ANIT may not trigger the formation of blood clots.

Based on the results of this study, ANIT induces the formation of blood clots in a dose dependent manner. Low levels of ANIT would indicate low levels of clotting due to the absence of serine proteases and essential clotting factors. Medium doses of clotting show the presence of kininogen that initiates blood clotting. Fibrinogen was found to be highest in 100mg/kg dose of ANIT. Fibrinogen plays a role in the final stages of blood clotting.

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